

UC Irvine

UC Irvine Previously Published Works

Title

Mechanism of charybdotoxin block of a voltage-gated K⁺ channel.

Permalink

<https://escholarship.org/uc/item/6q25z289>

Journal

Biophysical journal, 65(4)

ISSN

0006-3495

Authors

Goldstein, SA
Miller, C

Publication Date

1993-10-01

DOI

10.1016/s0006-3495(93)81200-1

Copyright Information

This work is made available under the terms of a Creative Commons Attribution License, available at <https://creativecommons.org/licenses/by/4.0/>

Peer reviewed

Mechanism of Charybdotoxin Block of a Voltage-Gated K⁺ Channel

Steve A. N. Goldstein* and Christopher Miller

Howard Hughes Medical Institute, Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts, USA

ABSTRACT Charybdotoxin block of a *Shaker* K⁺ channel was studied in *Xenopus* oocyte macropatches. Toxin on rate increases linearly with toxin concentration in an ionic strength-dependent fashion and is competitively diminished by tetraethylammonium. On rate is insensitive to transmembrane voltage and to K⁺ on the opposite side of the membrane. Conversely, toxin off rate is insensitive to toxin concentration, ionic strength, and added tetraethylammonium but is enhanced by membrane depolarization or K⁺ (or Na⁺) in the trans solution. Charge neutralization of charybdotoxin Lys27, however, renders off rate voltage insensitive. Our results argue that block of voltage-gated K⁺ channels results from the binding of one toxin molecule, so that Lys27 enters the pore and interacts with K⁺ (or Na⁺) in the ion conduction pathway.

INTRODUCTION

Charybdotoxin (CTX) is a 37-amino acid peptide toxin of known structure that inhibits several types of K⁺ channels with nanomolar affinity (Anderson et al., 1988; Bontems et al., 1992; Sugg et al., 1990). The mechanism by which CTX blocks one such channel, the high-conductance Ca²⁺-activated K⁺ (BK) channel of rat skeletal muscle, is known. A single CTX molecule binds to a receptor in the channel's outer vestibule, physically occluding its conduction pathway (Giangiacomo et al., 1992; MacKinnon and Miller, 1988). Site-specific mutants of CTX produced in *Escherichia coli* have recently allowed the study of the contribution of individual CTX residues to toxin binding in the BK channel (Giangiacomo et al., 1993; Park and Miller, 1992b; Stampe et al., 1992). Association of CTX into its receptor site is enhanced by weak, through-space electrostatic interaction of several positively charged amino acids on the toxin (Park and Miller, 1992b) and unidentified negatively charged carboxylates of the channel (MacKinnon and Miller, 1989a). Once bound, eight CTX residues interact intimately with the channel surface to create a high-affinity bimolecular complex (Stampe et al., manuscript submitted for publication). Addition of K⁺ to the internal solution speeds CTX dissociation, as if K⁺ ion, entering the pore from the inside, destabilizes the bound toxin (MacKinnon and Miller, 1988). This effect of K⁺ is enhanced by depolarization and is wholly mediated by a single positively charged residue on CTX, Lys27 (Park and Miller, 1992a). If this lysine is changed to an uncharged residue, toxin dissociation becomes completely independent of both K⁺ and applied voltage. The interaction of pore-associated K⁺ with bound CTX has provided additional evidence that the toxin receptor site of the BK channel is located near the external opening of the K⁺-selective conduction pathway.

Our interest in the nature of the CTX receptor site is motivated by its location near the K⁺ channel's catalytic machinery: the ion conduction pore. Insight into the structure of the channel's outer vestibule will place constraints on structural models of pore architecture and may have a direct bearing on channel function. However, the BK channel, which has provided the most detailed information about CTX interactions, is still poorly understood at the molecular level. We therefore undertook a study of a CTX-sensitive, voltage-gated K⁺ channel that is genetically manipulable, the molecular architecture of which has been extensively evaluated, and the CTX receptor determinants in which are known (Goldstein and Miller, 1992; MacKinnon and Miller, 1989b; MacKinnon et al., 1990). In this paper, we address a single question: Does CTX block the *Shaker* K⁺ channel in the same simple, pore-directed fashion known for the BK channel? Confirmation of such a mechanism would strengthen the rationale for extending a structure-function inquiry to this channel's CTX receptor site.

We find that CTX blockade of voltage-gated *Shaker* channels is, like block of Ca²⁺-activated BK channels, the result of a 1:1 stoichiometric binding interaction in the channel outer pore. Like BK channels, the voltage dependence of CTX block of the *Shaker* channels requires a positive charge on toxin position 27. Whereas electrostatic interactions influence toxin association and dissociation rates similarly for the two channel types, the voltage dependence of *Shaker* channel blockade differs in subtle ways. First, CTX block of *Shaker* channels exhibits a small inherent voltage dependence, as though a charge on the toxin moves through roughly 20% of the transmembrane potential drop when toxin binds. Second, Na⁺ ions on the opposite side of the membrane alter CTX (and tetraethylammonium) affinity by entering or traversing the *Shaker* pore. Finally, external K⁺ ions selectively alter the binding kinetics of some CTX isoforms in the outer *Shaker* channel vestibule.

MATERIALS AND METHODS

Electrophysiology

Expression of the CTX-sensitive, inactivation-removed *Shaker* K⁺ channel used in this work in *Xenopus* oocytes after cRNA injection has been described previously (Goldstein and Miller, 1992). Currents were recorded

Received for publication 14 May 1993 and in final form 24 June 1993.

Address reprint requests to Dr. Christopher Miller, Howard Hughes Medical Institute, Graduate Department of Biochemistry, Brandeis University, Waltham, MA 02254-9110

*Present address: Yale University School of Medicine, Boyer Center for Molecular Medicine, 295 Congress Avenue, New Haven, CT 06536-0812.

© 1993 by the Biophysical Society

0006-3495/93/10/1613/07 \$2.00

from outside-out patches without leak subtraction using an Axopatch 1-C (Axon Instruments, Foster City, CA). Bath solution was either 2 mM K (out): 2 mM KCl, 100 mM NaCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.1), and 25 μ g/ml bovine serum albumin, or 100 mM KCl (out): 100 mM KCl, 2 mM NaCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 10 mM HEPES (pH 7.1), and 25 μ g/ml bovine serum albumin. Internal pipette solution was 100 mM KCl (in) unless indicated to be 100 mM NaCl (in) or 100 mM LiCl (in) and contained 1 mM MgCl₂, 5 mM EGTA, and 10 mM HEPES (pH 7.1). All experiments were performed at 21–23°C.

To accomplish fast fluid exchange (<10 ms) at the patch surface, the electrode tip bearing the patch was positioned in the outflow port of a tubular track (0.05 inch wide) through which flowed various test solutions under solenoid control. Solution flow was constant during both control and blocking pulses and was gravity driven. Multipulse experiments were performed as follows. Wash-in: After four control pulses of 10–25 ms of toxin-free bathing solution, the solution was rapidly changed to one containing toxin, and brief test pulses were applied until equilibrium blockade was achieved. Wash-out: After four control pulses were collected at equilibrium in the presence of toxin, the switch to toxin-free solution was made and pulses were collected until full recovery was achieved. The same patch was always studied for a complete toxin wash-in and wash-out cycle. Time constants were determined by single-exponential fits.

Single-pulse experiments were performed as follows. Wash-in: After four control pulses of 450–750 ms of toxin-free solution, the solution was rapidly exchanged to one containing toxin, about 50 ms after the beginning of the test pulse. Three additional pulses were subsequently taken to establish that blocking equilibrium had been achieved. Wash-out was performed by a similar protocol, using a switch from toxin-containing to toxin-free solution. Time constants were determined by either single or double exponential fits, according to the relative rates of toxin kinetics and slow inactivation.

Production of recombinant CTX variants

CTX and its mutant isoforms were produced in *E. coli* and purified as previously described (Park et al., 1991; Stampe et al., manuscript submitted for publication). Each mutant was assayed on both *Shaker* and BK channels, and previously established criteria (Park and Miller, 1992b; Stampe et al., manuscript submitted for publication) for correct folding of toxin were checked. For two very weak variants used here (Y36A and K27Q), two-dimensional nuclear magnetic resonance was used to confirm directly the backbone folding pattern.

RESULTS

Recombinant CTX blocks a modified *Shaker* K⁺ channel with high affinity

The K⁺ channel designed for this work was modified in two ways (Goldstein and Miller, 1992) from the *Drosophila Shaker* B K⁺ channel (Schwarz et al., 1988). First, the channel was altered by a point mutation (F425G) in the pore-associated S5-S6 linker region. This mutation reveals a receptor site with an inhibition constant (K_i) for CTX of 75 pM (Table 1), 2000-fold higher affinity than that exhibited by the native channel (Goldstein and Miller, 1992). Second, the channel's NH₂-terminal inactivation domain was deleted ($\Delta 6-46$) (Hoshi et al., 1990), a maneuver that leaves CTX block unaffected. For all experiments, CTX isoforms were purified after expression in *E. coli* of a synthetic gene for CTX linked to a cleavable fusion protein carrier (Park et al., 1991; Stampe et al., manuscript submitted for publication).

TABLE 1 Blocking parameters for wild-type and mutant charybotoxins

Toxin	K_i (nM)	$k_{on} \times 10^{-6}$ (M ⁻¹ s ⁻¹)	k_{off} (s ⁻¹)
Multi-pulse protocol			
Wild-type	0.075 \pm 0.005	63 \pm 5	0.0047 \pm 0.0004
S10Q	0.69 \pm 0.05	149 \pm 18	0.085 \pm 0.006
R25Q			
2 K ⁺ (out)	0.95 \pm 0.05	23 \pm 2	0.021 \pm 0.007
Single pulse protocol			
R25Q			
100 K ⁺ (out)	1.7 \pm 0.2	126 \pm 5	0.22 \pm 0.02
K27M	330 \pm 8	103 \pm 3	34 \pm 1
K27N	590 \pm 80	90 \pm 7	70 \pm 3
K27Q	1,200 \pm 50	50 \pm 4	70 \pm 5
K27R	104 \pm 4	50 \pm 4	5.3 \pm 0.6
K27S	4500 \pm 400	7 \pm 1	36 \pm 5
R34Q	29 \pm 1	150 \pm 10	4.3 \pm 0.3
Y36A	154 \pm 2	83 \pm 3	13.5 \pm 0.6
Y36Q	226 \pm 18	160 \pm 5	37 \pm 2

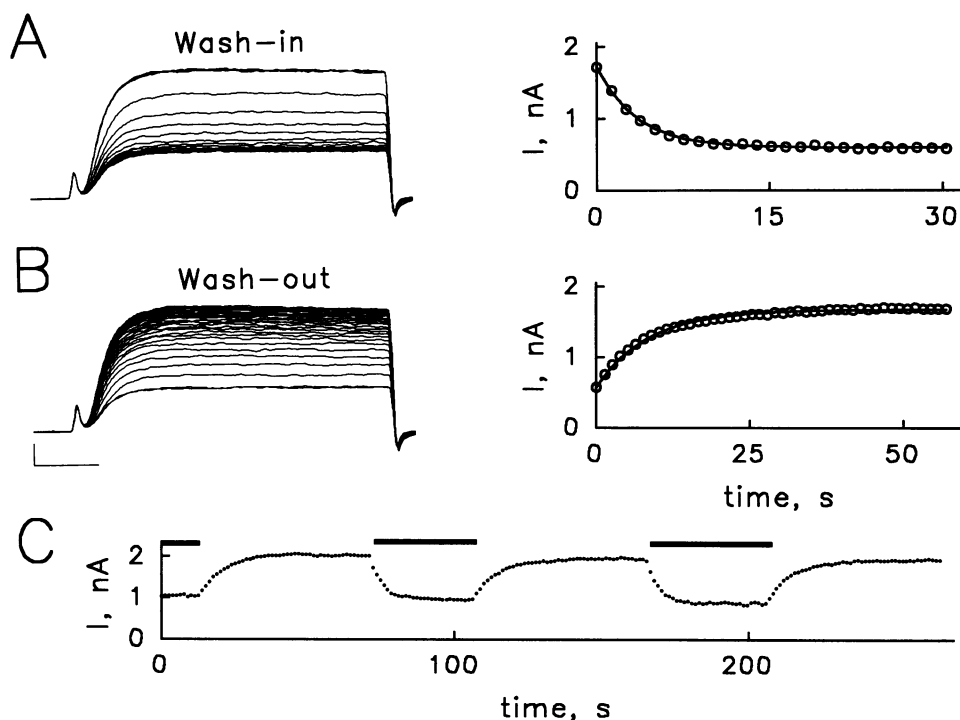
The inhibition constant (K_i), second-order association rate constant (k_{on}), and first-order dissociation rate constant (k_{off}) were determined for wild-type CTX, S10Q, and R25Q by the multipulse protocol and by the single-pulse protocol at 0 mV for R25Q and the remaining CTX mutants. The values shown are the mean \pm SE for 4–22 independent determinations. All experiments were performed with 2 mM KCl (out) and 100 mM KCl (in), except for R25Q, which was also studied in 100 mM KCl (out) and 100 mM NaCl (in) solutions as indicated.

Multipulse protocols to determine the kinetics of toxin blockade

Channels were expressed at high density by microinjection of cRNA into *Xenopus* oocytes (Goldstein and Miller, 1992). Macropatches were excised from oocytes in an outside-out configuration to expose the external face of the channels to toxin in the bath solution. For measurement of toxin kinetics, patches expressing 0.5 to 2.5 nA of K⁺ current were moved to the rapid-exchange perfusion chamber, where toxin could be applied or removed by rapid solution exchange (<10 ms). For each experimental value, kinetics were assessed in four or more patches that survived a complete wash-in and wash-out cycle and recovered to within 5% of preblock current levels. Such criteria are difficult to meet when studying toxins with slow, wild-type CTX dissociation rates (0.0047 s⁻¹), since each patch must be stable for over 20 min. Therefore, each multipulse experiment was first performed in several patches with wild-type CTX and subsequently repeated using the CTX variants S10Q or R25Q with off rates 5- to 20-fold higher than wild-type. A summary of the blocking characteristics of all CTX mutants used here is given in Table 1.

Fig. 1 shows a multipulse wash-in/wash-out experiment using S10Q. In multipulse experiments, patches were held at -100 mV and then repetitively briefly depolarized to 0 mV to assess unblocked current levels. In this experiment, pulses of 10 ms were collected at 1-s intervals. After four control pulses (Fig. 1 A), the patch was abruptly exposed to toxin, and the progressive block of K⁺ current to a new steady-state level was followed. Toxin block did not alter the time course of *Shaker* activation, since S10Q binds to both closed and open states of the channel. Plots of unblocked current over

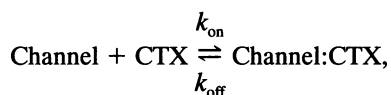
FIGURE 1 Blockade of K^+ current by CTX mutants. Outside-out patches expressing the channels were studied by the multipulse protocol in the fast perfusion chamber. In all experiments, patches were held at -100 mV and pulsed to 0 mV for 10 ms, with an interpulse interval of 1 s. (A) Successive pulses after wash-in of 1 nM S10Q and a plot of the relaxation of current measured at 9.5 ms into each test pulse with single-exponential fit superimposed. (B) Wash-out following experiment (A). Scale bars represent 0.25 nA and 2 ms. (C) A patch was subjected to repetitive application and removal of 1 nM S10Q (solid bars) under conditions identical to those above.



time are well fit by single exponentials for both toxin wash-in and wash-out (Fig. 1, A and B). Block was fully reversible upon wash-out (Fig. 1 C). We emphasize that full reversibility is observed for CTX and all 72 mutant toxins studied (data not shown).

Simple bimolecular blockade

Since toxin binding is reversible and does not alter channel gating, the time constants for relaxation to equilibrium block upon abrupt toxin exposure (and to unblocked current levels after toxin removal) reflect only the progress of the binding reaction. If CTX blockade of *Shaker* K^+ channels proceeds by the simple bimolecular reaction scheme



where k_{on} ($\text{M}^{-1}\text{s}^{-1}$) is the second-order association rate constant and k_{off} (s^{-1}) the first-order dissociation rate constant, experimentally measured time and rate constants will exhibit characteristic behavior. According to this scheme, the time constant for relaxation to equilibrium blockade is described by

$$\tau_{\text{in}} = (k_{\text{on}}[\text{CTX}] + k_{\text{off}})^{-1}, \quad (1)$$

and the time constant for relaxation to unblocked current levels following toxin removal is described by

$$\tau_{\text{out}} = (k_{\text{off}})^{-1}. \quad (2)$$

Fig. 2 shows the effects of increasing toxin concentration on block kinetics. As required by a simple bimolecular scheme,

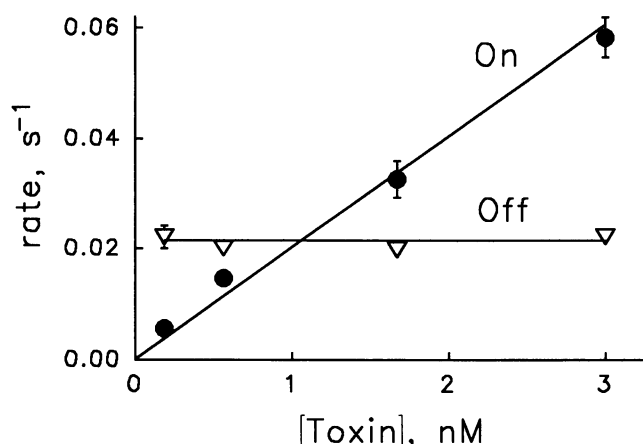


FIGURE 2 Effect of toxin concentration on blocking kinetics. The kinetics of blockade by R25Q was measured at the indicated toxin concentrations using the multipulse protocol. The apparent first-order rate constants for association (On, $k_{\text{on}}[\text{Tx}]$) and dissociation (Off, k_{off}) are plotted as a function of toxin concentration. Patches were held at -100 mV and pulsed to 0 mV for 25 ms, with an interpulse interval of 4 s. Each point represents the mean \pm SE of 4–7 experiments, each in a separate patch. The slope of the line through the first-order association rate constants corresponds to a second-order association rate constant k_{on} of $19 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$.

association rate increases linearly with toxin concentration, and dissociation rate remains constant. Thus, like block of BK channels, block of *Shaker* K^+ channels reflects the formation of a bimolecular toxin-channel complex.

The fraction of unblocked current at equilibrium (f_u) is also readily measured and related to these rate constants according to

$$f_u = k_{\text{off}} / (k_{\text{on}}[\text{CTX}] + k_{\text{off}}) \quad (3)$$

and

$$K_i = k_{\text{off}}/k_{\text{on}} \quad (4)$$

Thus toxin on rates, off rates, and inhibition constants can be determined from both wash-in or wash-out experiments. We used this overdetermination property as a check on kinetic parameters in all experiments.

Competitive inhibition between tetraethylammonium and toxin

Tetraethylammonium (TEA) ion slows the association of CTX into its BK channel receptor site but does not influence dissociation of toxin from its site (Miller, 1988). This is the demanded result if TEA and toxin bind to the pore in a mutually exclusive ("competitive") fashion. Fig. 3 shows that in *Shaker* channels as well, TEA is a pure competitive inhibitor of toxin binding. As TEA concentration is increased, the K_i for toxin increases linearly, entirely as a result of a decrease in toxin association rate (Fig. 3). Toxin off rate is unaffected by external TEA, as would be expected if TEA and toxin binding sites in the channel overlap.

Ionic strength effects on CTX block kinetics

CTX is a basic peptide carrying a net positive charge in aqueous solution of roughly +5 at neutral pH (Gimenez-Gallego et al., 1988). As a result, binding of CTX should be sensitive to through-space electrostatic forces. When the fixed negative surface charges on the external face of the BK channel are shielded by high ionic strength (Anderson et al., 1988), the association rate of CTX is diminished while toxin dissociation remains unaltered.

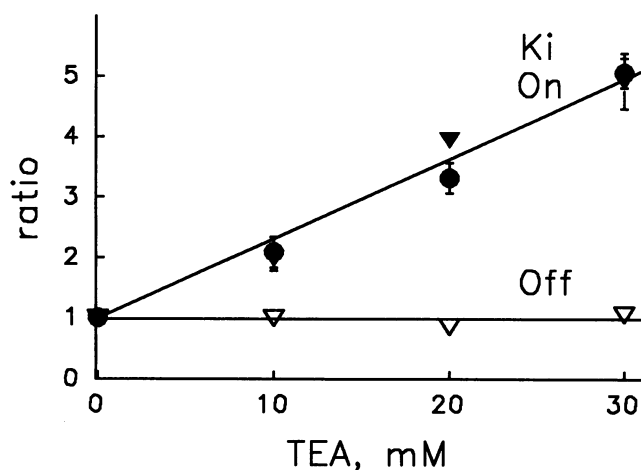


FIGURE 3 Effect of TEA on CTX kinetics. The kinetics of R25Q blockade was studied in the presence of varying concentrations of external TEA, as in Fig. 2. Each measured parameter— K_i (●), off-rate (▽), and reciprocal on-rate constant (●)—is normalized to the value without TEA (listed in Table 1). The bathing solution was 2 mM KCl (out), except where the Cl salt of TEA was substituted isotonicly for NaCl. Each point represents the mean \pm SE for 5–8 patches.

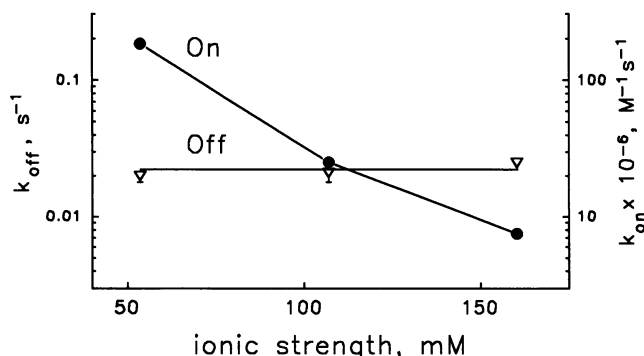


FIGURE 4 Effect of external ionic strength on toxin kinetics. The kinetics of R25Q blockade was assessed at three ionic strengths, as in Fig. 2. The second-order association rate constant (On , $k_{\text{on}} \times 10^{-6} \text{M}^{-1} \text{s}^{-1}$) and dissociation rate constant (Off , $k_{\text{off}} \times 10^{-3} \text{s}^{-1}$) are plotted on the ordinate for R25Q in the presence of increasing NaCl. The bathing solution was 2 mM KCl (out), where NaCl was altered to increase or decrease the ionic strength. Inclusion of sorbitol to maintain constant osmolarity did not alter measured kinetic parameters (data not shown). Each point represents the mean \pm SE for 4–10 patches.

Fig. 4 shows that the association of CTX into the *Shaker* channel toxin receptor site is also markedly sensitive to ionic strength. A threefold increase in ionic strength (from 50 to 150 mM) results in a 25-fold decrease in R25Q association rate, while dissociation of the toxin-channel complex is essentially unaltered. A negatively charged residue of the pore-associated S5-S6 domain, E422, has previously been shown to influence binding of a CTX isoform to *Shaker* K^+ channels in an ionic strength-dependent manner (MacKinnon and Miller, 1989b; Escobar et al., 1993). Some CTX variants also show variation of dissociation from the *Shaker* channels with external ionic strength, and this is the subject of ongoing study.

External K^+ influences CTX blocking kinetics

In contrast to CTX block of BK channels (Anderson et al., 1988), external K^+ specifically affects the binding of some CTX isoforms to *Shaker* channels. For example, when external K^+ is raised from 2 to 100 mM, association and dissociation of R25Q are speeded, 2- and 45-fold, respectively (Table 1). This sensitivity to external K^+ but not Na^+ suggests that this residue senses the occupancy of an external K^+ coordination site, possibly related to site(s) thought to mediate effects of external K^+ on gating of other K^+ channels (Shapiro and DeCoursey, 1991; Pardo et al., 1992).

Single-pulse protocols and blocking kinetics of low-affinity toxins

The CTX analogs described above exhibit off rates that are significantly slower than the rate of "slow" (or "C-type") inactivation in this channel (Hoshi et al., 1991). For this reason, the kinetics of blockade was studied by holding channels at a hyperpolarized voltage and assessing the fraction of unblocked channels by brief depolarizing pulses. Under

these conditions, CTX interacts with channels that are closed nearly all the time. In contrast, for weakly binding toxins with rapid off rates, it is possible to observe the kinetics of channel-toxin interaction within a single pulse. This capability allows the evaluation of block of open channels and permits study of the voltage dependence of toxin block, a mechanistically revealing characteristic. We therefore used a weakly binding CTX mutant, Y36A, to attack questions concerning the voltage dependence of block.

Fig. 5 A demonstrates that the fast-perfusion system exchanges the solution bathing a patch in less than 10 ms; this was judged using TEA, a rapid blocker, applied to the patch during a sustained 450-ms depolarizing test pulse. The wash-in and wash-out kinetics of Y36A are illustrated in Fig. 5 B; full equilibration of toxin occurs within the time of the depolarizing pulse, with time constants which are at least an order of magnitude faster than the most significant confounding kinetic process, slow inactivation. Relaxations are fit well by two exponentials representing the rate constants for block and slow inactivation.

Voltage dependence of toxin block

CTX mutants in which each of the toxin's nine charge-bearing residues was individually neutralized were used to study block of BK and *Shaker* channels (Park and Miller, 1992b; Goldstein and Miller, unpublished observations). For both channel types, only three of these residues—R25, K27, and R34—are crucial to toxin binding and blockade. The mutants produced by neutralizing each of these basic residues to glutamine (Table 1) showed weakened binding mainly due to increased off rates, as if these residues are intimately involved in toxin-channel contact. In the BK channel, Lys27 exclusively mediates the voltage dependence of CTX dissociation (Park and Miller, 1992a), and so we tested whether this residue displays this property in *Shaker* as well.

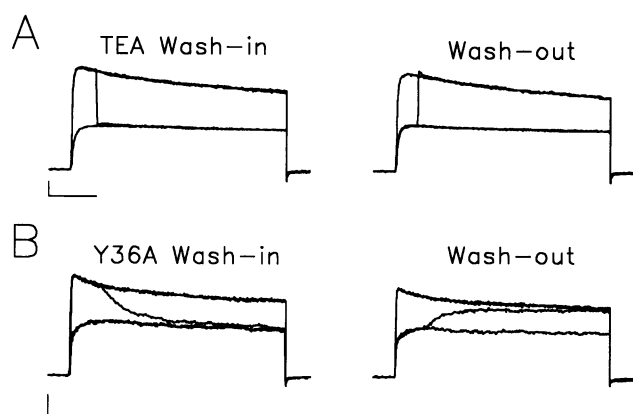


FIGURE 5 Single-pulse blocking kinetics for weak toxins. The single-pulse protocol was applied to follow the blocking kinetics of weak toxins at high concentrations. Patches were held at -100 mV and pulsed to 0 mV for 450 ms, with an interpulse interval of 1 s. (A) 20 mM TEA wash-in and wash-out. Blocking and unblocking were complete in less than 10 ms. (B) 100 nM Y36A wash-in and wash-out. The scale bars are 0.2 nA and 100 ms in both panels.

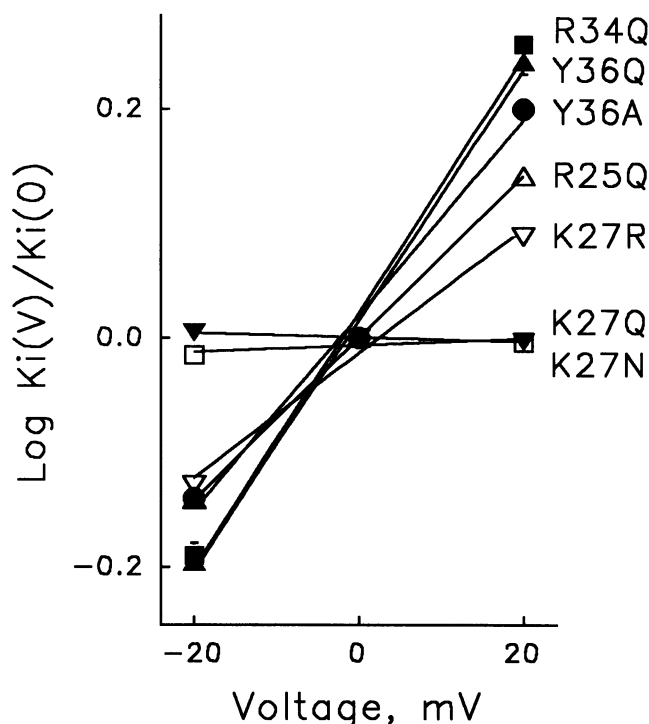


FIGURE 6 Voltage effects on equilibrium blockade by mutant CTXs. Inhibition constants (K_i) for each toxin were determined at the indicated voltages from the fraction of unblocked current at equilibrium (f_u) and are plotted as a ratio to their value at 0 mV. Conditions were as in Fig. 5 except for R25Q experiments, where the internal pipette solution was 100 mM NaCl (in) and the bathing solution 100 mM KCl (out). Each point represents the mean \pm SE for 5–10 patches that survived measurement of both block and unblock at all three voltages at least three times. Effective valence of block ($z\delta$) was determined for each toxin from the linear fits shown: $\log[K_i(V)/K_i(0)] = z\delta FV/RT$; these values are: R34Q, -0.60 ± 0.03 ; Y36A, -0.53 ± 0.02 ; R25Q, -0.41 ± 0.01 ; K27R, -0.31 ± 0.02 ; K27N, -0.01 ± 0.01 ; K27Q, -0.02 ± 0.02 ; Y36Q, -0.62 ± 0.02 . The absolute values for the inhibition constants (K_i) at 0 mV are reported in Table 1.

To study the effect of voltage on toxin blockade of *Shaker* channels we used the single-pulse protocol with several low-affinity CTX variants (Fig. 6). For these experiments, we contrast toxins maintaining a positive charge at position 27 with those in which Lys27 has been neutralized. As with BK channels, equilibrium block of *Shaker* is weakened by depolarization if position 27 carries a positive charge (R25Q, K27R, R34Q, Y36A, Y36Q). This voltage dependence is cleanly eliminated if Lys27 is neutralized to N, Q (Fig. 6), M, or S (data not shown). Transmembrane voltage exerts its effect on toxin affinity solely through the dissociation reaction, as Fig. 7 illustrates with Y36A. The toxin's off rate increases with depolarization, and its on rate remains constant, as is the case for CTX isoforms on the BK channel (MacKinnon and Miller, 1988; Giangiacomo et al., 1992).

Effect of internal cations on the voltage dependence of CTX block

In BK channels, CTX block is sensitive to transmembrane voltage only when internal K^+ is present to traverse the pore

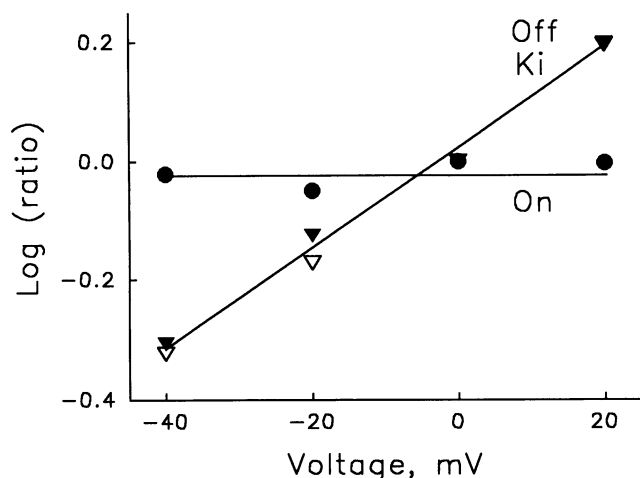


FIGURE 7 Voltage dependence of Y36A blockade. The kinetics of block by Y36A was studied at the indicated voltages, as in Fig. 5. Each parameter was measured at all voltages and normalized to its value at 0 mV; ∇ , k_{off} ; \bullet , k_{on} ; \blacktriangledown , K_i . Each point represents the mean \pm SE for 10 patches that survived measurement of block and unblock at all four voltages at least three times.

and interact with the charge on toxin Lys27 (Park and Miller, 1992a). When Na^+ is the only internal monovalent cation, voltage no longer affects CTX block of the BK channel (MacKinnon and Miller, 1988). But this is not the case for Y36A blockade of *Shaker* K^+ channels (Table 2). Here, when all internal K^+ is replaced by either Na^+ or Li^+ , voltage dependence of block is reduced but not eliminated. Since this voltage dependence still requires a positive charge at position 27 (Fig. 6), we conclude that the ϵ -amino group of Lys27 actually senses a small fraction, $\sim 20\%$, of the voltage drop across the pore.

This unexpected conclusion is supported by control experiments on external TEA blockade. With internal K^+ present, TEA block of *Shaker* channels is relieved by depolarization, in analogy to classic experiments by Armstrong (1971) on squid axon K^+ channels. As reported in Table 2, when internal K^+ is replaced by Li^+ , the voltage dependence of TEA block is lost; this result is understandable if we assume that the impermeant Li^+ ion cannot pass down the

TABLE 2 Effect of internal monovalent cation on voltage-dependent block

	$z\delta$		
	100 mM K^+ (in)	100 mM Na^+ (in)	100 mM Li^+ (in)
Y36A	-0.53 ± 0.02	-0.39 ± 0.01	-0.20 ± 0.01
TEA	-0.14 ± 0.02	-0.09 ± 0.02	0.00 ± 0.01

Inhibition constants (K_i) for Y36A and TEA were determined as in Fig. 7 and used to calculate effective valence ($z\delta$). The internal pipette solution was 100 mM XCl (in), where X is K, Na, or Li, and bathing solution was 2 mM KCl (out) for X = K and 100 mM KCl (out) for X = Na or Li as described in Materials and Methods. Each value represents the mean \pm SE for 5–10 patches that survived measurement of both block and unblock at all four voltages at least three times.

K^+ -selective pore far enough to destabilize TEA on its blocking site. Internal Na^+ , like K^+ , confers voltage dependence on TEA block, although the effective valence of block is lower with Na^+ than with K^+ (Table 2). These cation effects on TEA block argue that both K^+ and Na^+ are able to traverse the pore, whereas Li^+ cannot. Accordingly, we consider the voltage dependence of Y36A block of *Shaker* channels that remains in the presence of internal Li^+ to be inherent to the toxin itself.

DISCUSSION

We have described six mechanistic points about the interaction of CTX and a *Shaker* K^+ channel through the study of the association and dissociation of the toxin. CTX block of *Shaker* K^+ channels, in broad view, resembles block of Ca^{2+} -activated K^+ channels. First, CTX blockade of *Shaker* channels follows simple, reversible, bimolecular kinetics. This is true for both wild-type CTX and its mutant derivatives with equilibrium inhibition constants (K_i) varying over four orders of magnitude, from 75 pM to 4.5 μM . Second, CTX and TEA bind in a mutually exclusive fashion to the *Shaker* channel pore. Third, the association of CTX into its *Shaker* channel receptor site is aided by through-space electrostatic forces between the positively charged toxin and negatively charged residues on the channel surface. Fourth, CTX dissociation is accelerated by depolarization. Fifth, a critical toxin residue, Lys27, uniquely mediates the voltage dependence of toxin block. Sixth, CTX is destabilized by K^+ ions entering the channel from the opposite side. The results strongly imply that CTX blockade of these voltage-gated *Shaker* K^+ channels is, as with Ca^{2+} -activated K^+ channels, a simple, bimolecular, and pore-directed binding event.

While the above characteristics are reminiscent of the CTX blocking mechanism on BK channels several new aspects of this interaction are seen in the *Shaker* channel. First, Na^+ acts like K^+ in destabilizing bound toxin (as well as TEA), as though this poorly permeant ion traverses a substantial length of the pore. We are inclined, on the basis of TEA experiments, to conclude that Li^+ is impermeant to *Shaker* channels and does not interact with blockers through the pore. Given this picture, we interpret the small voltage dependence of toxin block with internal Li^+ as inherent to the toxin. This argues that Lys27 moves about 20% of the way down the potential drop as the toxin snuggles into its receptor site in the vestibule of the *Shaker* channel. The major (>2000 -fold) destabilization resulting from the relatively conservative mutation K27R, without loss of voltage dependence of block, supports the notion that position 27 interacts intimately with a "narrow" region of the *Shaker* channel pore surface (Table 1). This is in clear distinction to the BK channel where K27R blocks well (Park and Miller, 1992a) and appears to bind near, but about 4 Å away from, the central axis of symmetry (Stampe et al., manuscript submitted for publication). Another distinguishing characteristic of *Shaker* channel blockade is the strong, specific effect of external K^+ ion on both blocking and dissociation of R25Q. This effect,

which is not observed in the BK channel, suggests the presence of an external K^+ -selective site near the toxin receptor in the channel vestibule.

The conclusion that CTX block of this voltage-gated K^+ channel is pore directed is not surprising. Mutations in the *Shaker* S5-S6 pore-associated linker domain have been known for some years to alter scorpion toxin blockade (Goldstein and Miller, 1992; MacKinnon et al., 1990). Nevertheless, a rigorously supported view of blockade as a physical occlusion of the pore provides a compelling basis for studying the residues involved in the binding of toxin and channel. Thus, individual toxin residues have been identified as critical to CTX blockade of *Shaker* channels (Goldstein et al., unpublished observations), and these sites can cautiously be considered binding site candidates. Reciprocally, we have identified residues on the K^+ channel that are important to toxin block (Goldstein et al., unpublished observations). The challenge for future work is to identify intermolecular contact partners via complementary mutagenesis of CTX and *Shaker*. Such contact pairs, when combined with the known structure of CTX, will provide molecular landmarks for deducing the physical topography of the *Shaker* channel's external vestibule.

We are grateful to Drs. Per Stampe and Shimon Marom for advice throughout the course of these experiments and to Daniel Goldstein and Emmy Novick for their continued support. This research was supported in part by National Institutes of Health grants GM-31768 to C. M. and HL-02770 to S. A. N. G.

REFERENCES

- Anderson, C., R. MacKinnon, C. Smith, and C. Miller. 1988. Charybdotoxin inhibition of Ca^{2+} -activated K^+ channels. Effects of channel gating, voltage, and ionic strength. *J. Gen. Physiol.* 91:317–333.
- Armstrong, C. M. 1971. Interaction of tetraethylammonium ion derivatives with the potassium channels of giant axons. *J. Gen. Physiol.* 58:413–437.
- Bontems, F., B. Gilquin, C. Roumestand, A. Menez, and F. Toma. 1992. Analysis of side-chain organization on a refined model of charybdotoxin: structural and functional implications. *Biochemistry*. 31:7756–7764.
- Escobar, L., M. J. Root, and R. MacKinnon. 1993. The influence of protein surface charge on the bimolecular kinetics of a potassium channel peptide inhibitor. *Biochemistry*. 32:6982–6987.
- Gianguiacomo, K. M., M. L. Garcia, and O. B. McManus. 1992. Mechanism of iberiotoxin block of the large-conductance calcium-activated potassium channel from bovine aortic smooth muscle. *Biochemistry*. 31:6719–6727.
- Gianguiacomo, K. M., E. E. Sugg, M. Garcia-Calvo, R. J. Leonard, O. B. McManus, G. J. Kaczorowski, and M. L. Garcia. 1993. Synthetic charybdotoxin-iberiotoxin chimeric peptides define toxin binding sites on calcium-activated and voltage-dependent potassium channels. *Biochemistry*. 32:2363–2370.
- Gimenez-Gallego, G., M. A. Navia, J. P. Reuben, G. M. Katz, G. J. Kaczorowski, and M. L. Garcia. 1988. Purification, sequence, and model structure of charybdotoxin, a potent selective inhibitor of calcium-activated potassium channels. *Proc. Natl. Acad. Sci. USA*. 85:3329–3333.
- Goldstein, S. A. N., and C. Miller. 1992. A point mutation in a *Shaker* K^+ channel changes its charybdotoxin binding site from low to high affinity. *Biophys. J.* 62:5–7.
- Hoshi, T., W. N. Zagotta, and R. W. Aldrich. 1990. Biophysical and molecular mechanisms of *Shaker* potassium channel inactivation. *Science (Washington DC)*. 250:533–538.
- Hoshi, T., W. N. Zagotta, and R. W. Aldrich. 1991. Two types of inactivation in *Shaker* K^+ channels: effects of alterations in the carboxy-terminal region. *Neuron*. 7:547–556.
- MacKinnon, R., and C. Miller. 1988. Mechanism of charybdotoxin block of Ca^{2+} -activated K^+ channels. *J. Gen. Physiol.* 91:335–349.
- MacKinnon, R., and C. Miller. 1989a. Functional modification of a Ca^{2+} -activated K^+ channel by trimethylxonium. *Biochemistry*. 28:8087–8092.
- MacKinnon, R., and C. Miller. 1989b. Mutant potassium channels with altered binding of charybdotoxin, a pore-blocking peptide inhibitor. *Science (Washington DC)*. 245:1382–1385.
- MacKinnon, R., L. Heginbotham, and T. Abramson. 1990. Mapping the receptor site for charybdotoxin, a pore-blocking potassium channel inhibitor. *Neuron*. 5:767–771.
- Miller, C. 1988. Competition for block of a Ca^{2+} -activated K^+ channel by charybdotoxin and tetraethylammonium. *Neuron*. 1:1003–1006.
- Pardo, L. A., S. H. Heinemann, H. Terlau, U. Ludewig, C. Lorra, O. Pongs, and W. Stuhmer. 1992. Extracellular K^+ specifically modulates a rat brain K^+ channel. *Proc. Natl. Acad. Sci. USA*. 89:2466–2470.
- Park, C. S., and C. Miller. 1992a. Interaction of charybdotoxin with permeant ions inside the pore of a K^+ channel. *Neuron*. 9:307–313.
- Park, C. S., and C. Miller. 1992b. Mapping function to structure in a channel-blocking peptide: electrostatic mutants of charybdotoxin. *Biochemistry*. 31:7749–7755.
- Park, C. S., S. F. Hausdorff, and C. Miller. 1991. Design, synthesis, and functional expression of a gene for charybdotoxin, a peptide blocker of K^+ channels. *Proc. Natl. Acad. Sci. USA*. 88:2046–2050.
- Schwarz, T. L., B. L. Tempel, D. M. Papazian, Y.-N. Jan, and L.-Y. Jan. 1988. Multiple potassium-channel components are produced by alternative splicing at the *Shaker* locus in *Drosophila*. *Nature (Lond.)*. 331:137–142.
- Shapiro, M. S., and T. E. Decoursey. 1991. Permeant ion effects on the gating kinetics of the type L potassium channel in mouse lymphocytes. *J. Gen. Phys.* 97:1251–1278.
- Stampe, P., L. Kolmakova-Partensky, and C. Miller. 1992. Mapping hydrophobic residues of the interaction surface of charybdotoxin. *Biophys. J.* 62:8–9.
- Sugg, E. E., M. L. Garcia, J. P. Reuben, A. A. Patchett, and G. J. Kaczorowski. 1990. Synthesis and structural characterization of charybdotoxin, a potent peptidyl inhibitor of the high conductance Ca^{2+} -activated K^+ channel. *J. Biol. Chem.* 265:18745–18748.